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CHARACTERIZATION OF CALMODULIN FROM  
C<sub>6</sub> ASTROCYTOMA CELLS AND RAT CEREBRUM

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Jay Alan Gates

1986

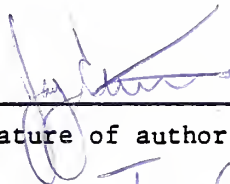
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CHARACTERIZATION OF CALMODULIN  
FROM C<sub>6</sub> ASTROCYTOMA CELLS AND RAT CEREBRUM

A Thesis Submitted to the Yale University  
School of Medicine in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Medicine

by  
Jay Alan Gates  
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\*CaM, calmodulin



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## ABSTRACT

### Characterization of Calmodulin From C<sub>6</sub> Astrocytoma Cells and Rat Cerebrum

Jay Alan Gates

1986

Calmodulin (CaM) regulates many processes associated with cellular proliferation and CaM antagonists can inhibit cell growth. To determine whether or not exploitable pharmacological differences exist between a normal and malignant tissue, we purified CaM by preparative polyacrylamide gel electrophoresis (PAGE) from rat cerebrum and the C<sub>6</sub> malignant astrocytoma cell line and compared the biochemical and pharmacological properties of the two preparations. The concentration of biologically active CaM in the heated, acidified supernatants was similar, being  $37 \pm 14$  ng/mg protein in cerebrum compared to  $26 \pm 7$  ng/mg protein in C<sub>6</sub> cells. The proteins eluted similarly from a preparative PAGE column, co-migrated in denaturing SDS-PAGE gels, and showed similar shifts of mobility when calcium was present during electrophoresis.

We tested the sensitivity of the two preparations of CaM to several classes of antagonists. No significant difference in the sensitivities to any of the classes of drugs was demonstrable when assayed by the inhibition of a CaM-sensitive cyclic nucleotide phosphodiesterase. We conclude that at least in one malignant cell line, the pharmacological properties of CaM are similar to those of normal tissue.



## INTRODUCTION

### Calmodulin and normal cellular proliferation

Calmodulin (CaM\*) is a heat and acid-stable, low molecular weight protein which was first described independently by Cheung (1) and Kakiuchi (2) as an activator of cyclic AMP phosphodiesterase. Teo and Wang showed that the same protein, in a purified form, bound calcium at multiple sites; they suggested that it was the complex of activator protein and calcium which was responsible for increased cyclic AMP phosphodiesterase activity (3). CaM, as the major intracellular calcium receptor, has since been shown to be involved in many processes that are necessary for normal cellular proliferation (for reviews see 4-7). For example, CaM changes in concentration as cells enter and traverse the cell cycle. Chafouleas et al demonstrated that CaM levels are elevated two-fold at late G<sub>1</sub> and/or early S phases during the growth cycle of CHO-K<sub>1</sub> cells (8). Sasaki and Hidaka showed that CaM concentrations increased after CHO-K<sub>1</sub> cells entered the S phase and reached a maximum level at the late G<sub>2</sub> or early M phase (9). It has been shown by MacManus and coworkers that following partial hepatectomy, CaM increases in regenerating hepatocytes, an increase which coincides with the time when calcium is required for the initiation of DNA synthesis (10). CaM is also known to stimulate the polymerization and depolymerization of microtubules. Welsh et al used indirect immunofluorescence techniques to demonstrate the localization of CaM to the spindle of mitotic

\*The abbreviations used are: CaM, calmodulin; EGTA, ethylene glycol bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; FBS, fetal bovine serum; PMSF, phenylmethylsulfonylfluoride; TEMED, N,N,N',N'-tetramethylethylenediamine; PAGE, polyacrylamide gel electrophoresis; PDE, phosphodiesterase; SDS, sodium dodecylsulfate. W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide



cells (11). Marcum and coworkers expanded on Welsh's work and showed that during anaphase CaM was restricted to the chromosome-to-pole region of the mitotic apparatus and that CaM was involved in the control of calcium dependent inhibition and reversal of microtubule assembly in vitro (12). Welsh et al also showed that while both CaM and tubulin are components of the mitotic apparatus, their localizations throughout mitosis are distinctly different and specific (13). Job and coworkers showed that a subpopulation of microtubules was almost totally resistant to the effect of calcium or CaM alone but disassembled rapidly in the presence of both at apparently physiological concentrations (14). It is also known that CaM modulates enzymes necessary for generation of cellular energy. Anderson and Cormier showed that CaM from the pea plant, Pisum sativum c., modulates NAD kinase activity in a calcium-dependent fashion (15). CaM also modulates enzymes necessary for control of cyclic nucleotide metabolism (1,16). Uzunov and Weiss demonstrated that of the several molecular forms of cyclic adenosine 3',5'-monophosphate phosphodiesterase, only one was really susceptible to activation by CaM (17). It has also been shown that CaM is capable of stimulating adenylate cyclase activity (18,19).

#### Calmodulin in states of abnormal cellular proliferation

In states of abnormal cellular proliferation, as occurs in transformed or malignant cells, control of calcium and CaM mediated events is altered (for reviews see 7,20-22). Watterson and coworkers found that CaM comprised approximately 1.3% of the soluble protein in homogenates of chicken embryo fibroblasts infected and transformed by Rous sarcoma virus as compared to 0.3% of the soluble protein in homogenates of normal fibroblasts (23). Balk et al have observed that transformed chick embryo fibroblasts have the ability to



grow in calcium concentrations that are 10-fold lower than that required for proliferation of normal cells (24). Chafouleas and coworkers used radioimmunoassay techniques to demonstrate a two-fold increase in the intracellular concentration of CaM in mammalian tissue culture cells transformed by oncogenic viruses (25). By comparing fast-growing Morris hepatoma tissue lines to normal adult liver tissue, Criss and Kakiuchi demonstrated that both CaM activity and total calcium are increased in the neoplastic tissue (20). Wei et al have demonstrated, in slowly and rapidly growing hepatomas, that increased CaM levels are directly proportional to tumor growth rate (26). It should be noted that Veigl and coworkers believe that the variation in CaM content, observed between normal and transformed cells, is complex and may in part depend on culture conditions; they suggest that the apparent inability of transformed cells to down-regulate CaM levels is a function of differing abilities of normal and transformed cultures to respond to cell-to-cell contact and/or transmediated cellular regulation signals (27).

The inhibition of abnormal cellular proliferation by CaM antagonists

Drugs that share the property of CaM antagonism are known to inhibit the growth of many malignant cell lines (for review see 7). For example, Speaker et al used the macrophage-like cell line J774 (established from a murine reticulum cell sarcoma) to demonstrate the inhibition of growth and Fc-mediated phagocytosis by the CaM antagonist trifluoperazine (28). In several studies Hait and coworkers have shown that the CaM antagonists calmidazolium, melittin, pimozide, trifluoperazine, and chlorpromazine inhibit the growth and/or clonogenicity of human and murine leukemic cells, and rat astrocytoma cells; they have demonstrated that the potency of these drugs, as inhibitors of cell growth, correlates directly with their activity as inhibitors of CaM (29-32). Ito and Hidaka were able to demonstrate a significant anti-tumor effect when



they gave intraperitoneal injections of W-7, a calmodulin antagonist, to mice implanted with solid Sarcoma-180 (33). Wei et al, using a soft agar clonogenic assay, showed that trifluoperazine and W-7 inhibited colony formation in the human breast cancer cell line, MDA-MB-231 (34). MacNeil and coworkers concluded that W-7 and prochlorperazine markedly inhibited DNA synthesis in B-16 melanoma cells, and that the inhibitory effect of these CaM antagonists was reversed by the presence of exogenous CaM (35).

CaM antagonists have also been shown to, at least partially, overcome tumor cell resistance to certain chemotherapeutic agents. For example, Tsuruo et al have shown that certain CaM inhibitors, as well as calcium antagonists, enhance the cytotoxic effects of vincristine and adriamycin in resistant P388 leukemic cells; this effect was attributed to enhanced intracellular levels of vincristine and adriamycin, possibly due to inhibition of their outward transport (36). In a similar system, Ganapathi and Grabowski demonstrated that trifluoperazine markedly enhanced cellular retention of, and sensitivity to, adriamycin only in adriamycin-resistant tumor sublines and not in adriamycin-sensitive lines (37).

#### The existence of novel calmodulins

Recently, novel CaMs have been identified in several organisms including Tetrahymena (38), Dictyostelium (39), and trypanosomes (40). These altered forms of the molecule differ structurally, functionally, and antigenically (41) from mammalian CaM. For example, Yazawa and coworkers have described a Tetrahymena CaM (differing from bovine brain CaM by 11 amino acid substitutions and one deletion) which is unique in its ability to activate Tetrahymena guanylate cyclase (38). Ruben et al have isolated CaM from the trypanosome parasite which is structurally and functionally distinct from that of host tissue (40). Marshak and coworkers demonstrated that Dictyostelium CaM was



able to activate bovine brain cyclic nucleotide PDE as effectively as bovine brain CaM; however, it could activate pea NAD kinase 5 times greater than bovine brain CaM (39). One of the key distinctions between Dictyostelium CaM and bovine brain CaM is that Dictyostelium CaM's lysine 115 is not trimethylated and has been shown by Gregori et al to be a ubiquitin conjugation site (42); these investigators have demonstrated that mammalian CaM, which contains trimethyllysine at the 115 position, does not form conjugates with ubiquitin and thus, unlike dictyostelium CaM, is "protected" from ubiquitin-dependent, protein degradation pathways.

Furthermore, recent evidence suggests that different CaMs might exist within the same organism. Putkey and coworkers have isolated a distinct "CaM-like" gene from chicken which results in a protein product similar to normal chicken CaM, yet it contains 16 amino acid substitutions and activates rat brain PDE with slightly different kinetics (43). Siegel and Wright report the isolation of a "CaM-isotype" from beef testes which activates cyclic nucleotide PDE equally as well as does "standard" beef testes CaM, yet which stimulates a distinctly different protein methylation pattern in rat liver cytosolic fractions (44).

#### The potential use of calmodulin antagonists as antineoplastic agents

Based on the above findings, and the remarkable genetic diversity of tumor cells, we postulated that alterations in CaM during malignant transformation could occur which might help explain the ability of tumor cells to thrive in lower concentration of calcium compared to their normal counterparts (45) or which might provide a target for selective inhibition by drugs (7). Because of the mounting interest in the potential use of CaM antagonists as antineoplastic agents alone (7,29-34), or to modulate resistance to known chemotherapeutic agents (36,37), it would be important to know whether



exploitable pharmacological differences exist between the molecule from a malignant cell and that of its normal counterpart.

#### Isolation of CaM by preparative polyacrylamide gel electrophoresis

We also tested the feasibility of preparing CaM by preparative-PAGE, a simple, rapid technique for the isolation and purification of other proteins (17). Therefore, we have established conditions for purifying CaM from normal and malignant cells by preparative-PAGE and report the application of these methods to the C<sub>6</sub> astrocytoma cell line and normal cerebrum.



## MATERIALS AND METHODS

### Cell culturing techniques

C<sub>6</sub> rat astrocytoma cells were obtained from the American Type Culture Collection (Rockville, MD). This glial cell tumor of rats was originally induced by intravenous injection of N-nitrosomethylurea. Cells were cultured at 37°C in a 5% atmosphere of CO<sub>2</sub> in plastic flasks in monolayers in Dulbecco's modification of Eagle's medium supplemented with 10% FBS. Cells were passed twice weekly and were shown to be free of contamination by routinely assaying for mycoplasma, bacteria and fungi.

### Isolation and Purification of CaM from C<sub>6</sub> astrocytoma cells and rat cerebrum

C<sub>6</sub> astrocytoma cells, at a concentration of approximately 100,000 cells per ml, were seeded on to 250 x 250 x 20 mm, plastic plates (Nunc Corp., Denmark) and were incubated at 37°C in Dulbecco's modification of Eagle's medium with 10% FBS in a 5% atmosphere of CO<sub>2</sub> for 3-4 days until confluent (doubling time approximately 18 hrs). Cells were harvested with a rubber spatula, pooled, and centrifuged at 450 x g for 20 minutes at 2°C. Medium was discarded and cells were washed with cold, fresh medium. Cells were homogenized in 1.5 volumes of phosphate buffer containing 90 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM EGTA, 0.5 mM PMSF, (pH 6.0) for twenty seconds with a Tisumizer<sup>R</sup> (Tekmar, Cincinnati, OH). The homogenate was then sonicated 10 pulses at 50 watts, (Tekmar<sup>R</sup>, Cincinnati, OH) in the presence of additional PMSF (0.5 mM). All procedures were carried out on ice.

Female Sprague-Dawley rats (Charles River, Kingston) were maintained on normal rat chow and distilled water. Rats weighing approximately 200 grams were decapitated with a Harvard Dispatcher. The cerebrum was removed, weighed, rinsed with distilled water, and patted dry. Tissue was suspended in 1.5



volumes of the phosphate buffer (pH 6.0) and was homogenized and sonicated as above.

The sonicated material was then placed in a boiling water bath for five minutes. The coagulated proteins were removed by centrifugation at  $3400 \times g$  for 20 minutes at  $2^{\circ}\text{C}$  (Model J-6B centrifuge, Beckman, Palo Alto, CA). The supernatant was readjusted to a pH of 6.0, placed in a boiling water bath for five minutes then recentrifuged. A portion of the supernatant was dialyzed overnight (against 50 mM Tris, 0.2 mM  $\text{CaCl}_2$ , pH 7.4) for determination of CaM activity and concentration of protein. The remaining supernatant was concentrated by ultrafiltration (YM5 membrane, mol. wt. cut off of 5,000, Amicon, Danvers, MA). Sucrose was added to the concentrate to yield a final concentration of 0.32M and was applied to a 345 mm x 25 mm gel filtration column (G-75 Super Fine Sephadex, Pharmacia, Piscataway, NJ) pre-equilibrated with elution buffer containing 5mM TRIS-base, 0.1 mM EGTA, 1 mM mercaptoethanol, (pH 7.4). 1.5 ml fractions were collected overnight at  $2^{\circ}\text{C}$  and the first 70 fractions were assayed for calmodulin activity the following morning. Fractions containing calmodulin activity were pooled and concentrated by ultrafiltration (Amicon, YM5).

Calmodulin was further purified by a preparative PAGE. A 10% gel column (Shandon Southern Instruments, Inc., Sewickley, PA) was prepared with a gel buffer containing 39 mM  $\text{NaH}_2\text{PO}_4$ , 123 mM  $\text{Na}_2\text{HPO}_4$ , 0.46% TEMED, and 1 mM EGTA (pH 7.0); electrolyte buffer contained 88 mM  $\text{NaH}_2\text{PO}_4$ , 23 mM  $\text{Na}_2\text{HPO}_4$ , 640 mM glycine, and 1 mM EGTA (pH 6.0). Partially purified CaM (1.5 ml of C<sub>6</sub> astrocytoma or rat cerebrum post-Sephadex concentrate) containing 0.32 M sucrose was applied to the gel. Electrophoresis was started at 25 mA for the first hour and maintained at 60 mA thereafter, using a constant current power supply (Bio-Rad Laboratories, Model 500/200, Richmond, CA). Protein was eluted with 100



mM TRIS buffer containing 0.1 mM calcium (pH 7.6) at a flow rate of 0.3 ml/minute. The entire procedure was carried out in a cold-room and the column was continuously cooled by passing refrigerated coolant through an outer sleeve and an inner finger. Each fraction was collected for twenty minutes, diluted 1:10, and assayed for CaM activity. Fractions containing CaM activity were pooled and concentrated by ultra-filtration (Amicon YM-5). Aliquots of concentrated material were stored at  $-70^{\circ}\text{C}$  without loss of activity for up to 6 months.

#### Assay of CaM and effects of CaM antagonists

The activity of calmodulin was determined by its ability to activate a CaM-sensitive form of cyclic nucleotide phosphodiesterase which was prepared from mouse cerebrum as previously described (17). The activity of cyclic AMP phosphodiesterase was determined by the luciferin-luciferase method (46). The assay mixture contained 50 mM glycylglycine buffer myokinase (0.25 unit), pyruvate kinase (0.50 unit), 25 mM ammonium acetate, 3 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , 0.4 mM cyclic AMP, 5 mM dithiothreitol, 0.26 mM phosphoenolpyruvate, 1 nM ATP (pH 8.0) and phosphodiesterase in a total volume of 100 microliters. Concentrations of phosphodiesterase and time of incubation were chosen to insure linearity of the assay. Using our preparations of phosphodiesterase and CaM, there was at least a 4 fold activation of the enzyme in all experiments. Quantitation of biologically active CaM in each sample was determined by comparing its activation of phosphodiesterase to the activation of phosphodiesterase by known quantities of a purified preparation of CaM from bovine brain (Calbiochem, LaJolla, CA).

In these experiments, three to five concentrations of each tissue sample were assayed for CaM activity to determine the range in which the activity was



linear with respect to concentration of tissue. Comparisons between the tissues were carried out on the same day using freshly prepared tissue in the same assay.

The effect of antagonists on CaM-dependent activity of phosphodiesterase was measured by the ability of various concentrations of drug to inhibit the activation of phosphodiesterase in the presence of 2 units of CaM. One unit of activity was defined as the amount of calmodulin required to achieve 50% of the maximum activation of the enzyme. The effects of CaM antagonists and their vehicles on the standard curve were determined and corrected as necessary. Comparisons between the tissues were done in the same assay.

### Analytical Electrophoresis

SDS PAGE was performed as described by Laemmli (47). A 16% acrylamide gel (pH 8.9) was used with sample buffers containing 0.11 M Tris, 0.11 M sucrose, 0.2% SDS and either 5 mM EGTA or 5 mM calcium chloride (pH 7.5). Electrolyte buffer contained 0.43 M glycine, 0.05 M TRIS, 0.1% SDS (pH 8.7). Proteins were visualized by the use of Coomassie staining. Molecular weight standards were obtained from Pharmacia (Piscataway, NJ).

### Protein determinations and statistical analysis

Protein concentrations were determined by the method of Lowry (48) and statistical significance by Student's t-test.

### Drugs

Trifluoperazine and chlorpromazine were gifts from Dr. Alfred Brown (Smith Kline Beckman, Philadelphia, PA): Fluphenazine was a gift from Dr. Fred Gorelick; Flunarazine, Melittin and the histones, H<sub>1</sub> and H<sub>2</sub>B were purchased from Sigma Co. (St. Louis, MO): calmidazolium was kindly provided by Dr. James Long, Janssen Pharmaceutical Company (Piscataway, NJ). Calmidazolium was dissolved in dimethylsulfoxide; the effect of this vehicle was determined for



and subtracted from all parts of the assay. All other drugs were dissolved in distilled water.

#### Miscellaneous

PMSF, 5' adenosine-monophosphate, cyclic AMP, and firefly luciferin-luciferase were obtained from Sigma Co. (St. Louis, MO); myokinase and pyruvate kinase were obtained from Boehringer-Mannheim (New York, NY); bovine brain calmodulin was obtained from Calbiochem (LaJolla, CA); Spectrapor<sup>R</sup> dialysis tubing with a m.w. cutoff of 3,500 was obtained from Spectrum Medical Ind., Inc. (Los Angeles, CA) and was prepared by boiling in double-distilled water. The remaining reagents were electrophoretic or reagent grade and were purchased from commercial sources.



## RESULTS

### Concentration of biologically active CaM from C<sub>6</sub> astrocytoma cells and cerebrum

The concentration of biologically active CaM in the supernatant fraction was similar in C<sub>6</sub> astrocytoma cells ( $26 \pm 7$  ng/mg protein,  $n = 3$ ) to that of cerebrum ( $37 \pm 14$  ng/mg protein,  $n = 4$ ) ( $p > 0.5$ ).

### Purification of CaM from C<sub>6</sub> astrocytoma and cerebrum

CaM from the acid-heat treated supernatant was further purified by gel filtration and preparative PAGE. Both preparations eluted identically from the gel filtration column (data not shown). This material was pooled and concentrated to 1.5 ml then applied to the preparative PAGE apparatus. Figure 1 shows the electrophoretic profiles of CaM emerging from the preparative PAGE. CaM from both tissues eluted in a single major peak of activity after 4 hours of electrophoresis in fractions 10 to 30. The material from each source was found to be homogeneous under denaturing conditions (Figure 2).

Table 1 shows the results of purification of CaM. Following acid and heat precipitation, there was similar CaM present in C<sub>6</sub> astrocytoma cells and rat cerebrum. Gel filtration resulted in a 2 fold purification of the molecule from both sources but more importantly reduced the concentration of contaminating nucleotides which otherwise interfere with the luciferin-luciferase assay. Following preparative PAGE of the concentrated material, the recovery of CaM varied between experiments but was in the range of 40%-70%. Overall, in several experiments, the recovery of CaM from cerebrum was greater than the recovery from the C<sub>6</sub> cells.



### Characterization of CaM from C<sub>6</sub> astrocytoma and cerebrum

The preparations of purified CaM were further characterized according to their ability to migrate in polyacrylamide gels under denaturing conditions. Figure 2 demonstrates that the electrophoretic mobility of the CaM prepared from C<sub>6</sub> astrocytoma cells was similar to that prepared from rat cerebrum and had an apparent m.w. of 15,000. Both migrated identically to CaM from bovine brain purchased from a commercial source. Figure 2 also shows that all preparations of CaM showed similar shifts in electrophoretic mobility in the presence of calcium.

### Effect of CaM antagonists on CaM purified from C<sub>6</sub> astrocytoma cells and rat cerebrum

Table 3 shows that the effect of CaM antagonists on CaM purified from C<sub>6</sub> astrocytoma cells and CaM purified from rat cerebrum was similar. Calmidazolium and melittin, were the most potent compounds studied (IC<sub>50</sub> values of 10-14 nM and 12-15 nM respectively). The basic peptides, histone H<sub>1</sub> and histone H<sub>2</sub>B were slightly less potent (IC<sub>50</sub> of 22-36 nM). Flunarazine, a piperazine derivative was more potent than the phenothiazines (IC<sub>50</sub> 3 µM). Fluphenazine and trifluoperazine were intermediate in potency (IC<sub>50</sub> 5-8 µM and 9 µM respectively) and chlorpromazine was the least potent inhibitor studied (IC<sub>50</sub> of 16-20 µM).



## DISCUSSION

These studies demonstrate the feasibility of purifying CaM by preparative PAGE and also demonstrate the similarities between CaM purified from rat cerebrum and a malignant astrocytoma cell line. The concentration of biologically active CaM was not significantly different in the two preparations. This observation confirms earlier work by Brostrom and Wolff (49) who found similar concentrations and similar biochemical characteristics of CaM in C<sub>6</sub> cells compared to that of brain. However, others have found that the concentration of CaM was increased in certain tumors and transformed cell lines (reviewed in 7,22). Since cerebrum contains many cell types which conceivably vary in their concentration of CaM (50), while the C<sub>6</sub> line is a malignancy of astrocytes, purified preparations of astrocytes might be necessary to demonstrate significant differences in concentrations of CaM.

The CaM isolated from cerebrum and C<sub>6</sub> astrocytoma cells were also qualitatively similar. For example, they had the same estimated m.w. by gel filtration (data not shown) and by SDS-PAGE (Figure 2); they showed identical shifts of mobility in the presence of calcium (Figure 2) and; they had similar sensitivity to inhibition by antagonists (Table 2).

We studied several different types of CaM antagonists to optimize the chances of demonstrating pharmacological differences between the CaMs. For example, the phenothiazines require calcium-dependent hydrophobic, geometric and ionic interactions with CaM (51) while the histones may be more dependent on the ionic properties of the molecules (52). Flunarazine may be a tissue selective inhibitor of CaM-stimulated phosphodiesterase (53). However, as shown in Table 2, we found no significant differences in the sensitivity of the preparations of CaM to any of the antagonists. The similar pharmacological



properties of the two CaM's is further evidence for their structural equivalence.

Recently, oncomodulin, a unique calcium-binding protein resembling  $\beta$ -parvalbumin has been found exclusively in tumor cells and human placenta (54). We have not identified this protein in our preparations. This can be explained by the recently observed phenomena that unlike CaM, oncomodulin is a far more potent activator of CaM-sensitive phosphodiesterase from heart than of phosphodiesterase from brain (55,56) and might go undetected in our assay.

This study also demonstrated that preparative PAGE is a rapid and simple technique for purifying CaM. This procedure was based on earlier work on the purification of cyclic nucleotide phosphodiesterase by Uzunov and Weiss (17). These investigators demonstrated the separation of CaM from phosphodiesterase using preparative PAGE, but were unable to separate the CaM from other proteins. To overcome this, we used a higher percentage acrylamide gel (10% vs 7.5%) and a lower pH buffer system to slow the migration of other proteins out of proportion to the smaller, more acidic CaM molecule. This resulted in excellent separation of CaM from contaminating proteins (Table 2 and Figure 2). Although the final products appear homogenous on SDS PAGE, the use of two dimensional electrophoresis and isoelectric focusing or high pressure liquid chromatography might prove helpful in determining ultimate purity. The entire procedure takes less than 48 hours including the initial gel filtration step which removed the high concentration of nucleotides present in the malignant cells which interfere with the luciferin-luciferase assay. Since many investigators assay CaM by other techniques, it is likely that direct preparative PAGE of the acid-heat treated soluble supernatant would give excellent results that could be completed in one day.



The 70% yield and approximately 20 fold purification from the already partially purified soluble supernatant is comparable to standard methodology (57-59). The overall yield was in the range of 20 mg of CaM per kg of original tissue.

CaM purified by this technique was authenticated by; 1) its identical migration to that of bovine brain CaM in SDS gels (Figure 2); 2) its characteristic shift in electrophoretic mobility in the presence of calcium (Figure 2); 3) its ability to activate phosphodiesterase and; 4) its inhibition by CaM antagonists (Table 2).

The recovery of CaM from C<sub>6</sub> cells compared to that from cerebrum was generally lower after preparative-PAGE. It seems unlikely that the differences were due to contaminating proteases because of the presence of excess concentration of the protease inhibitors PMSF and EGTA. It is also possible that the CaM of tumor cells remained tightly associated with a CaM-binding protein and migrated differently during electrophoresis. However, the presence of the calcium-chelator, EGTA, during all steps of the purification should have allowed for separation of CaM from most of its binding sites.

The pharmacological properties of CaM from a normal and malignant cell line have not been previously compared. The similarities demonstrated in the present studies may have further implications. For example, others have shown that hydrophobic, cationic molecules can inhibit the growth of malignant cells in vitro (7,21,29-32,34) and in vivo (33) and have correlated this effect with the potency of the agents as CaM antagonists (30). Furthermore, CaM antagonists were shown to enhance the cytotoxicity of bleomycin (60,61) and modulate the resistance of certain cells to adriamycin and vincristine (36,37). These results have led to the inclusion of phenothiazines in clinical cancer chemotherapeutic trials (62). However, the inability to demonstrate



pharmacological differences between CaM from a normal tissue and a malignant cell line raises questions regarding potential selectivity of drug action. This does not rule out the possibility that alterations of CaM exist in other malignant tissues, or that selectivity could be achieved through variations in uptake or distribution of the drug. The use of preparative PAGE should provide a simple technique for comparing the molecule from other normal and malignant cell lines to determine if exploitable differences might exist.



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## LEGENDS TO TABLES

**TABLE 1** Purification of CaM from C<sub>6</sub> Astrocytoma and rat cerebrum

CaM was prepared from C<sub>6</sub> astrocytoma cells and rat cerebrum as described in Methods. Fractions under a single peak of CaM activity (isolated by gel filtration or preparative PAGE) were obtained, pooled, concentrated, and assayed as described in methods.

**TABLE 2.** Effect of Antagonists on the Activity of CaM from C<sub>6</sub> Astrocytoma Cells and Rat Cerebrum

CaM and phosphodiesterase were purified as described in Methods. The effect of antagonists and their vehicles was determined in the presence of 2U of CaM. The range of concentration of drugs was; trifluoperazine 1-128  $\mu$ M, fluphenazine 3-100  $\mu$ M, chlorpromazine 6-100  $\mu$ M, calmidazolium 0.001-0.2  $\mu$ M, melittin 0.001-0.5  $\mu$ M, histone H1 0.01-1  $\mu$ M, histone H<sub>2</sub> 0.01-1  $\mu$ M, and flunarazine 1-100  $\mu$ M. IC<sub>50</sub> was defined as the concentration of agent necessary to inhibit the activation of phosphodiesterase by 50%. Each IC<sub>50</sub> value was determined from individual dose-response curves run in triplicate. There was less than a 5% inhibition of phosphodiesterase by drugs when CaM was omitted from the assay.



TABLE 1

Purification of CaM from C<sub>6</sub> Astrocytoma and Rat Cerebrum

| <u>C<sub>6</sub> Astrocytoma</u> | <u>CaM (ng)</u> | <u>Protein (μg)</u> | <u>Concentration of<br/>Biologically Active CaM<br/>(ng CaM/μg protein)</u> | <u>% Yield</u> | <u>Purification<sup>1</sup></u> |
|----------------------------------|-----------------|---------------------|-----------------------------------------------------------------------------|----------------|---------------------------------|
| Homogenate (5 gm tissue)         |                 |                     |                                                                             |                |                                 |
| Acid-heat supernatant            | 146,000         | 4902                | 30                                                                          | -              | -                               |
| Post-Sephadex G-75               | 138,000         | 2286                | 60                                                                          | 94             | 2                               |
| Post-Prep PAGE                   | 55,000          | 138                 | 398                                                                         | 37             | 13                              |
|                                  |                 |                     |                                                                             |                | 24                              |
| <u>Rat Cerebrum</u>              |                 |                     |                                                                             |                |                                 |
| Homogenate (4 gm tissue)         |                 |                     |                                                                             |                |                                 |
| Acid-heat supernatant            | 110,000         | 3600                | 31                                                                          | -              | -                               |
| Post-Sephadex G-75               | 99,000          | 1680                | 59                                                                          | 90             | 2                               |
| Post-Prep PAGE                   | 79,000          | 96                  | 823                                                                         | 70             | 28                              |

<sup>1</sup>As compared to specific activity of acid-heat supernatant



TABLE 2

Effect of Antagonists on CaM Purified from C<sub>6</sub> and  
rat cerebrum

| <u>DRUGS</u>             | <u>C6</u>                   | <u>Rat Cerebrum</u> |
|--------------------------|-----------------------------|---------------------|
|                          | <u>IC<sub>50</sub> (μM)</u> |                     |
| Phenothiazines           |                             |                     |
| Trifluoperazine          | 8.6                         | 9.2                 |
| Fluphenazine             | 5.0                         | 8.5                 |
| Chlorpromazine           | 16.0                        | 20.0                |
| Peptides                 |                             |                     |
| Melittin                 | 0.01                        | 0.02                |
| H <sub>1</sub> Histone   | 0.02                        | 0.03                |
| H <sub>2</sub> B Histone | 0.05                        | 0.04                |
| Other                    |                             |                     |
| Calmidazolium            | 0.01                        | 0.01                |
| Flunarazine              | 2.5                         | 3.3                 |



## LEGENDS TO FIGURES

- Figure 1.** Preparative polyacrylamide gel electrophoresis of CaM from C<sub>6</sub> astrocytoma cells and rat cerebrum. 1-2 mg of protein was applied to the 10% polyacrylamide gel column and collected as described in methods. Broken line indicates the activity of phosphodiesterase in the absence of added CaM.
- Figure 2.** SDS-polyacrylamide gel electrophoresis of CaM purified from C<sub>6</sub> astrocytoma cells, rat cerebrum, and bovine brain. CaM was purified from C<sub>6</sub> astrocytoma and rat cerebrum by preparative PAGE as described in Methods. 5 µg of protein was applied to each lane of the 16% gel in a buffer containing either 5 mM EGTA (-) or 5 mM CaCl<sub>2</sub> (+). Molecular weight standards included ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000).



PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS  
OF CALMODULIN FROM C<sub>6</sub> ASTROCYTOMA CELLS  
AND RAT CEREBRUM

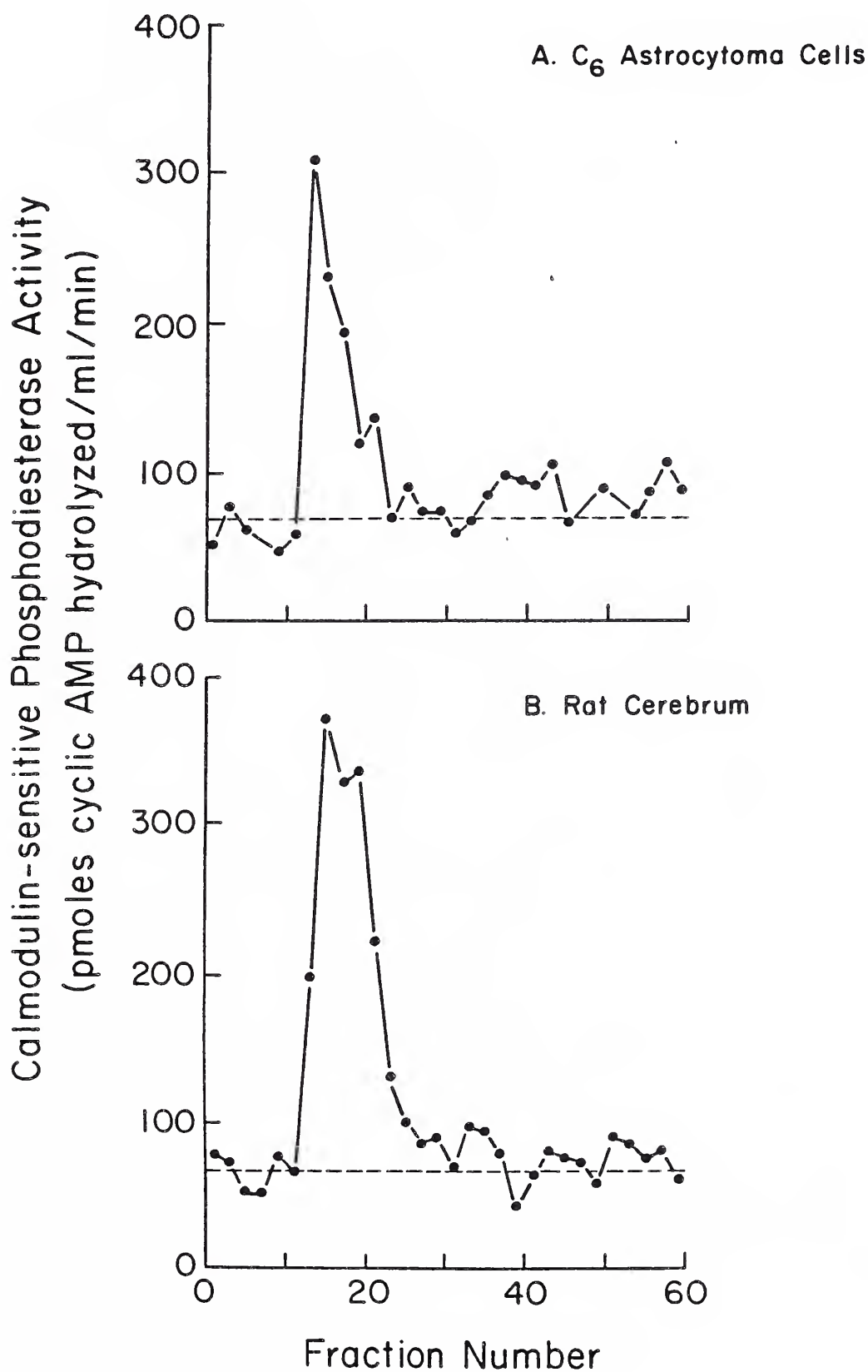


FIGURE 1



# ELECTROPHORETIC MOBILITY OF VARIOUS PREPARATIONS OF CALMODULIN IN 16% SDS-PAGE

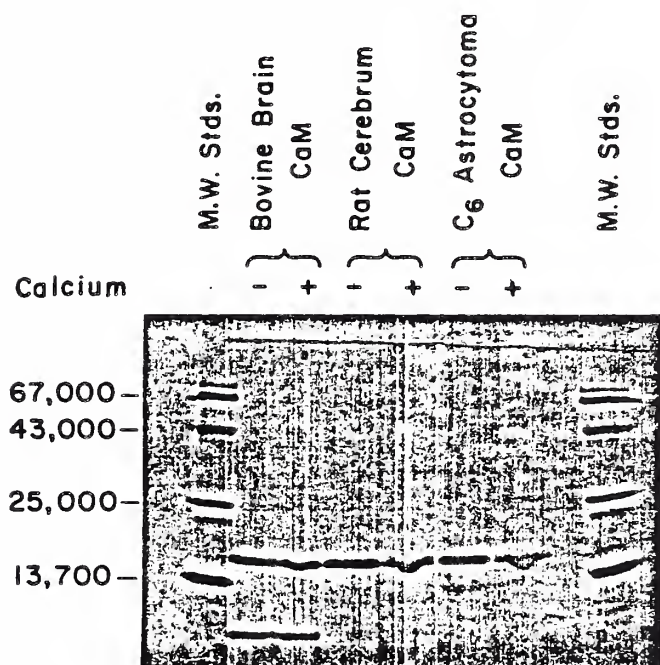


FIGURE 2









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